PROJECT REPORT

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DETAILED METHODS OF ANALYSIS FOR RESIDUE OF (2-CHLOROETHYL) PHOSPHONIC ACID (ETHEPHON) IN MILK AND COW LIVER, MUSCLE, KIDNEY AND FAT TISSUES

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SUMMARY:

Methods of analysis for possible residues of (2-chloroethyl) phosphonic acid (Ethephon) in cow milk and in liver, kidney, muscle and fat tissues are presented. For liver, kidney and muscle the method consists of weighing ground tissue samples into polyethylene bottles, freezing, and freeze drying the samples, followed by extraction with cold acidic methanol containing dimethyl tartrate. The methanol extract is filtered, concentrated under reduced pressure, and cooled to remove fats. Following centrifugation and subsampling a second precipitation step using hexane:ethyl acetate solution removes polar constituents. The extract is concentrated to a small volume and methylated with ethereal diazomethane. The methylated ethephon is separated on a florisil or silica gel column. The eluent is concentrated and analyzed by gas-liquid chromatography using a capillary column and flame photometric detection employing a phosphorus filter.

Fat tissue and milk are also freeze dried in preparation for extraction. These samples are extracted with warm acidic methanol. After cooling the extract in an ice bath, solids are removed by filtration. The filtrate is concentrated, subsampled and methylated. This sample is partitioned into water and then extracted from the aqueous solution with ethyl acetate. The ethyl acetate is concentrated and gas chromatographed. Milk samples are chromatographed as described in the other tissue method. Fats may be chromatographed with either capillary or packed columns.

Recoveries from samples fortified with 0.1 ppm ethephon were: Muscle, 54-73%; liver 54-80%, kidney 94-104%, fat 80-100% and milk 76-96%.

RESEARCH AND DEVELOPMENT DEPARTMENT
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Equipment

- Polyethylene graduated centrifuge tubes, 15 ml.
- Polyethylene bottles, 2 oz. screw-capped.
- Polyethylene pipettes, 1 ml graduated in 0.1 ml units.
- Blender.
- Freeze-dryer, Virtis 10-145 MR-BA or equivalent.
- Homogenizer, high-speed, Sorvall Omni Mixer or equivalent.
- Rotary evaporator, Buchii or equivalent.
- Vacuum pump or aspirator capable of maintaining a vacuum of 25-26 inches of water.
- Centrifuge.
- Graduated cylinders 25 and 100 ml.
- Buchner funnels.
- Whatman filter paper.
- Filtration flasks, 125 ml.
- Round bottom flasks 100 ml with standard 24/40 tapered joint.
- Graduated centrifuge tubes, 15 ml.
- Glass pipettes, 1, 5, and 10 ml with 0.1 ml graduations.
- Glass chromatography tubes, 10 mm I.D. X 30 cm length with Teflon stopcock.
- Gas chromatograph HP5880A with flame-photometric detector.
- Magnetic stirrer/heater

Reagents

- Hexane

- Ethylacetate Omnisolv EM Science

- Methanol

- Florisil 100/200 mesh J.T. Baker.

- Silica Woelm 63-200, Woelm Pharma

- Celite 545 Fisher Scientific

- Dimethyl tartrate solution - dissolve 10g reagent grade dimethyl tartrate in 100 ml methanol.

- Hydrogen chloride methanolic solution - A 3 molar solution of hydrogen chloride is prepared by bubbling the gas into methanol. The molarity of the solution is determined by titrating with 0.1M sodium hydroxide. A 1 ml aliquot of the methanolic hydrogen chloride in 10 ml of water containing phenolphthalein indicator is used.

- Diazomethane solution - Prepare from N-methyl-N-nitroso-p-toluene sulfonamide (Diazold, Aldrich Chemical Co.) according to manufacturer's instructions. Observe all safety precautions. Do not store over potassium hydroxide or other desiccants. Store tightly capped in freezer.

- Sodium chloride aqueous solution - dissolve 37g of reagent grade sodium chloride in 100 ml of water.

Reagents (Cont'd)

- Standard ethephon solutions - Weigh 10.0 mg of ethephon into a 15 ml graduated polypropylene centrifuge tube. Add 10.0 ml methanol and shake until dissolved. Transfer 1.0 ml to a second polypropylene tube using a 1 ml polyethylene pipet. Adjust volume to 10.0 ml with methanol and mix. Using this 0.1 mg/ml solution repeat one to ten dilution steps to obtain 0.01 mg/ml solution. Another serial dilution results in 1 µg/ml solution. This final solution may be used for fortification of tissue samples for determining recoveries. A polypropylene pipet must be used for dispensing this solution (see Note 1).

- Standard dimethylethephon solutions - Weigh 10.0 mg ethephon into a 15 ml graduated polypropylene centrifuge tube. Add 2 ml methanol and shake until ethephon is completely dissolved. Add diazomethane solution until a permanent yellow color is obtained. Remove excess diazomethane by using a gentle stream of nitrogen until solution is colorless. Adjust volume to 10.0 ml with methanol. Make four serial 10:1 dilutions using ethyl acetate as the solvent (either glass or plastic may be used following methylation for transferring solutions). The last two dilutions which contain 1 µg/ml and 0.1 µg/ml (acid equivalent) dimethylethephon are useful for gas chromatographic calibration.

PROCEDURE:

Sample Preparation (All tissues and milk)

1) Cut tissue into small pieces and freeze using dry ice.

2) Place approximately 50g hard frozen tissue plus 50 to 100g dry ice into a blender.

3) Grind until all non-fiberous particles are smaller than 0.3 cm.

4) Allow dry ice to sublime in freezer overnight.

5) Weigh 5g of tissue sample or 10 ml of whole milk into a 2 oz. polyethylene bottle. Hard freeze the samples by placing the vial on powdered dry ice.

6) Freeze dry the sample. (Note 7)

PROCEDURE FOR MUSCLE, LIVER AND KIDNEY TISSUE

Extraction, Filtration, and Concentration

1) Transfer tissue to a stainless steel blending flask. Fortification of samples for determination of recoveries should be done at this point. Care should be exercised to dispense the spiking solution onto the tissue and not on the walls of the flask (Note 1).

2) The blending flask is placed into a methanol-ice bath contained in a 400 ml beaker. Cold methanol, 20 ml (Note 2), 1.1 ml of 3M methanolic hydrogen chloride, and 0.25 ml of dimethyl tartrate solution was added to the blending flask.

3) This mixture is blended for 30-60 seconds with an Omnimixer while the flask is partly submerged in an ice bath.

4) This mixture is vacuum filtered through a 1 cm thick pad of prewashed celite in a Buchner funnel (Note 3).

5) The residual solids are transferred back to the stainless steel flask and an additional 20 ml cold methanol, 1.1 ml 3M methanolic hydrogen chloride and 0.25 ml of dimethyl tartrate solution is added.

6) Steps (3) and (4) are repeated.

7) The total filtrate is transferred to a 100 ml round bottom flask and concentrated to approximately 10 ml using a rotary evaporator and vacuum of 25 inches of water. A water bath temperature of 40-45°C is used (Note 2).

8) The methanol solution is transferred to a 15 ml graduated centrifuge tube (GCT). This solution is cooled to -10 to -12°C using a methanol-ice bath. Total volume is adjusted to 10 ml.

Precipitation Steps and Methylation

1) The solids are precipitated by centrifugation.

2) A 2.0 ml aliquot of the supernatant solution is transferred to a GCT and concentrated under a nitrogen stream to 1 ml.

3) Add 10 ml of a 3:10 hexane:ethyl acetate solution to a separate GCT. The 1 ml methanol extract (equivalent to 1g tissue) is added to the hexane:ethyl acetate solution. Wash the methanol containing tube with some of the hexane:ethyl acetate solution to insure a quantitative transfer (Note 4). (A precipitate forms on addition of the methanol extract.)

4) The solution is cooled in an ice bath and then centrifuged.

5) The solution is transferred to a GCT and concentrated under nitrogen to 1 ml. Addition of 0.1 ml of 3M methanolic hydrogen chloride is made.

Precipitation Steps and Methylation (Cont'd)

6) Ethereal diazomethane, 5 ml, is added to the GCT in a fume hood.

7) This solution is allowed to sit at room temperature for 5 minutes. Ethyl acetate, 2 ml, is added. The solution is concentrated under a nitrogen stream to 1 ml.

Florisil Separation for Liver and Muscle Extracts

1) Hexane, 4 ml, is added to the methylated extract.

2) Using hexane as the solvent, florisil is slurry packed in a glass chromatography tube, 1 X 30 cm, to a height of 15 cm.

3) The solvent is allowed to drip out until no solvent remains above the florisil. The extract is added to the top of the column.

4) Solvent is allowed to drip through the column until all the extract has passed on the florisil.

5) The column is then eluted with the following solvents (Note 5).

   a) 15 ml of 4:1 hexane:ethyl acetate.
   b) 50 ml of 2:1 hexane:ethyl acetate.
   c) 30 ml of 3:2 hexane:ethyl acetate.
   d) 50 ml of 2:3 hexane:ethyl acetate.

   This final solution contains 0.5 ml of 3M methanolic hydrogen chloride/100 ml solution.

6) Solvent D is collected in a 100 ml round bottom flask and concentrated under vacuum using a rotary evaporator and a water bath at 30°C to 5 to 10 ml.

7) The concentrate is transferred to a GCT (Note 6) and further concentrated to 2.0 ml under nitrogen. This solution represents a equivalent of 0.5g tissue/ml and is ready for gas chromatographic analysis.

SILICA GEL COLUMN SEPARATION FOR KIDNEY EXTRACTS

1) Add 2 ml of hexane to the concentrated extract.

2) Using ethyl acetate as the solvent, silica gel is slurry packed in a glass chromatography tube, 1 x 30 cm to a height of 15 cm.

3) The solvent is allowed to drip out until all the extract has passed onto the silica gel.

4) The column is eluted with the following solvents:

   A) 25 ml of ethyl acetate
   B) 35 ml of a mixture consisting of 85 ml ethyl acetate, 15 ml methanol, 2 ml of 3 M methanolic hydrogen chloride.

   The first 10 ml of the "B" solvent is discarded. The next 25 ml of solvent "B" is collected in a round bottom flask.

5) Concentrate this solution to approximately 10 ml. Add an additional 10 ml of ethyl acetate and concentrate to 5-10 ml.

6) Transfer to a graduated centrifuge tube and concentrate under N_2 to 2 ml. This solution represents an equivalent of 0.5 g tissue/ml and is ready for gas chromatographic analysis.

PROCEDURE FOR ETHEPHON RESIDUE ANALYSIS OF MILK AND FAT SAMPLES FOLLOWING FREEZE DRYING

EXTRACTION OF FREEZE DRIED SAMPLES

(1) Transfer freeze-dried sample to 125 ml Erlenmeyer flask. Fortification of control samples for recovery data should be carried out at this point.

(2) Add 20 ml of methanol, 1.0 ml of 3 M methanolic hydrogen chloride and a magnetic stirring bar to the flask. The flask is heated for 15-20 min. in a 55-60°C water bath while stirring.

(3) The flask is removed from the bath. At this point 40 ml of ethyl acetate is added to the milk extract. No ethyl acetate is added to the fat extract. The flask is placed in an ice bath for 10-15 minutes.

(4) Vacuum filter the solution through a celite pad in a Buchner funnel. Wash the pad with 3-5 ml of cold methanol.

(5) Concentrate the filtrate in a 100 ml round bottom flask on rotary evaporator under a vacuum to 5-10 ml. Transfer the solution to a graduated centrifuge tube. Concentrate the solution to 4 ml under a nitrogen gas stream. Wash the round bottom flask with 6 ml ethyl acetate to bring the volume in the tube to 10 ml. Mix the ethyl acetate and extract.

PRECIPITATION, SUBSAMPLING AND METHYLATION

1) Cool the sample tube in an ice bath.

2) Centrifuge to precipitate solids.

3) Take a 1.0 ml sample of milk extract and a 2 ml sample of fat extract and transfer it to another centrifuge tube.

4) Add 0.1 ml of 3 M methanolic hydrogen chloride

5) Methylate with ethereal diazomethane. Allow this solution to react for approximately 15 minutes.

6) Add 5 ml of ethyl acetate.

7) Concentrate under nitrogen gas stream to 1 ml.

PARTITION WITH WATER

1) Add 2 ml of hexane to the methylated sample.

2) Add 2 ml of deionized water containing 3-4 drops of 6 M hydrochloric acid. Shake and then centrifuge to separate phases.

3) Remove all the upper layer to a second tube using a transfer pipet. Repeat Step 2 making a second aqueous extraction.

4) Combined the aqueous extracts and 2 ml of aqueous sodium chloride solution (37g/100 ml).

5) Extract this aqueous solution with 3 x 6 ml of ethyl acetate, combining the ethyl acetate extracts in a 50 ml Erlenmeyer flask.

6) Dry the ethyl acetate extracts with a small quantity of anhydrous sodium sulfate.

7) Using a transfer pipette, transfer the ethyl acetate to a graduated centrifuge tube and concentrate to 2.0 ml under a nitrogen gas stream. These samples are ready for gas chromatograpic analysis (see Note 8).

Gas Chromatographic Analysis (Note 8)

1) Analysis of 1.1 µl of the extract was carried out on a Hewlett-Packard model 5880A chromatograph using a flame photometric detector in the phosphorus mode. Operating parameters were:

Capillary Column: 15M X 0.32 mm I.D. fused silica capillary column with 0.25 µm film thickness of bonded supelcowax 10.

Column Temperatures: Initial 50°C with level 1 program rate 10°C/min. with final temperature 160°C. Level 2 program rate 30°C/min. to 220°C held for 3 minutes.

Flow: 1 ml/min.

Attenuation: 2 3

Chart Speed: 1 cm/min.

Retention Time: See Chromatograms

Packed Column: 8' x 2 mm I.D. glass 6% DEGS-PS on 100/120 mesh chromosorb chromosorb G

Flow: 30 ml/min.

2) Analysis: The analysis was by peak area using standard dimethyl ethephon solution with each set of samples to determine the quantitative response of the integrator.

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Notes

1. It has been demonstrated that used glassware absorbs ethephon very strongly from dilute solutions. The use of silanized or polyethylene labware overcomes this problem. Therefore, it is necessary that either silanized or polyethylene labware (the latter is recommended) be used to prepare solutions of ethephon standards. In the presence of other polar materials such as extracts of tissues and crops glassware does not present this problem. It should be noted that surface absorption is not a problem once ethephon has been methylated.

2. It has been demonstrated that ethephon is not stable in the presence of liver and kidney extracts even in acid conditions at room temperature. By maintaining a temperature of less than 0°C using an ice bath containing methanol the extraction and filtration of muscle, liver, and kidney can successfully be carried out. The concentration of the filtrate should be at as low a pressure as is safe with the equipment available. A water bath temperature of no greater than 45°C to remove the methanol is recommended. Before and after the concentration step the solution should be at -10°C which is attained with the methanol-ice bath.

3. The celite pad was prepared in the Buchner funnel by washing the celite with 20 to 40 ml methanol. The filtrate was discarded and the filtration flask washed with an additional small volume of methanol. The fat in the cold methanol may cause some slowing of the filtration. Scraping the surface of the celite pad clear of the build up restores the filtrate flow. Since it is desirable to maintain a cold solution as much as possible, reasonable filtration rates are required.

4. The 1 ml methanol extract is transferred with a disposable pipette to the larger volume 3:10 hexane:ethyl acetate. The addition in this way prevents the inclusion of ethephon in the precipitate formed on the addition.

5. Florisil activity will vary depending on the moisture content. The solvent sequence described should be checked with dimethyl ethephon to be sure that it is eluting in the described fraction.

6. The graduated centrifuge tubes commonly available are not correctly calibrated. Since the final volume is 2 ml, this volume should be determined for the graduated centrifuge tube to contain the extract for gas chromatographic analysis.

7. Freeze drying tissue samples and milk does not result in loses of radiolabeled ethephon. This result has previously been reported for other ethephon analyses. In the present case we have confirmed these results by fortifying several milk sample with ^{14}C-Ethephon and freeze drying the samples. The radiocarbon extracted from these freeze dried samples accounted for 98% of that used for fortification.

Notes (continued)

8. Chromatograms of representative analyses of each tissue and milk are presented. The fat analyses using both packed and capillary columns are included. The capillary columns peteriorate with the analyses of these extracts. Increased usefulness of these column may be obtained by leaving the oven temperature at 210°C overnight. All the samples may be analyzed by packed column; However, for kidney and milk a sensitivity of 0.1 ppm may be very difficult to obtain since the ethephon peak appears as a shoulder on the solvent front.

TABLE 1

RESULTS FROM THE RESIDUAL ANALYSIS OF CONTROL MILK AND FAT, LIVER, MUSCLE, AND KIDNEY CONTROL TISSUES FORTIFIED WITH ETHEPHON

Tissues	Fortification (ppm)	% Recovery	% Average Recovery
Liver	0.5	69	
	0.1	54, 66, 70, 73	66%
Muscle	0.5	78	
	0.1	54, 66, 68, 80	64%
Kidney	0.5	86	
	0.1	94, 100, 100, 104	100%
Fat	0.5	80	
	0.1	80, 80, 98, 100	90
Milk	0.5	61	
	0.1	76, 88, 92, 96	88

FIGURE 1

CHROMATOGRAM OF DIMETHYL ETHEPHON (DME) STANDARD

SOLUTION 0.22 ng

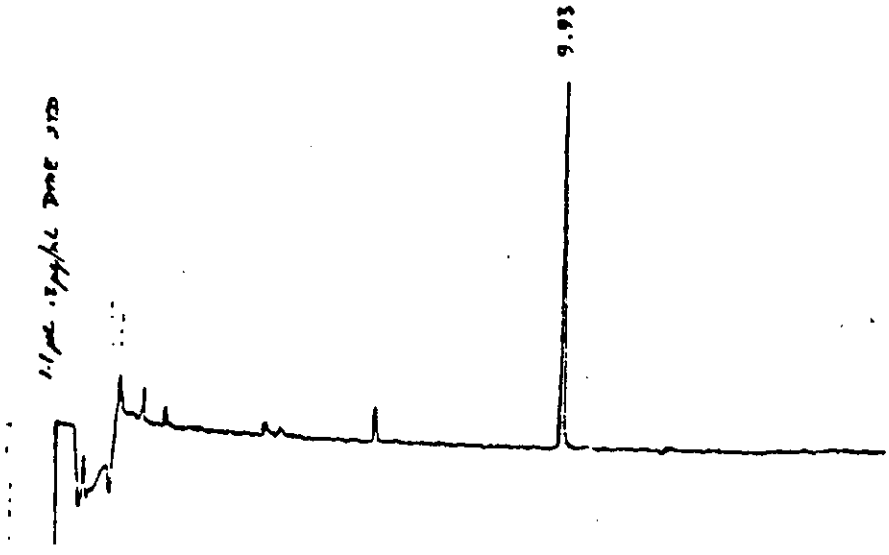
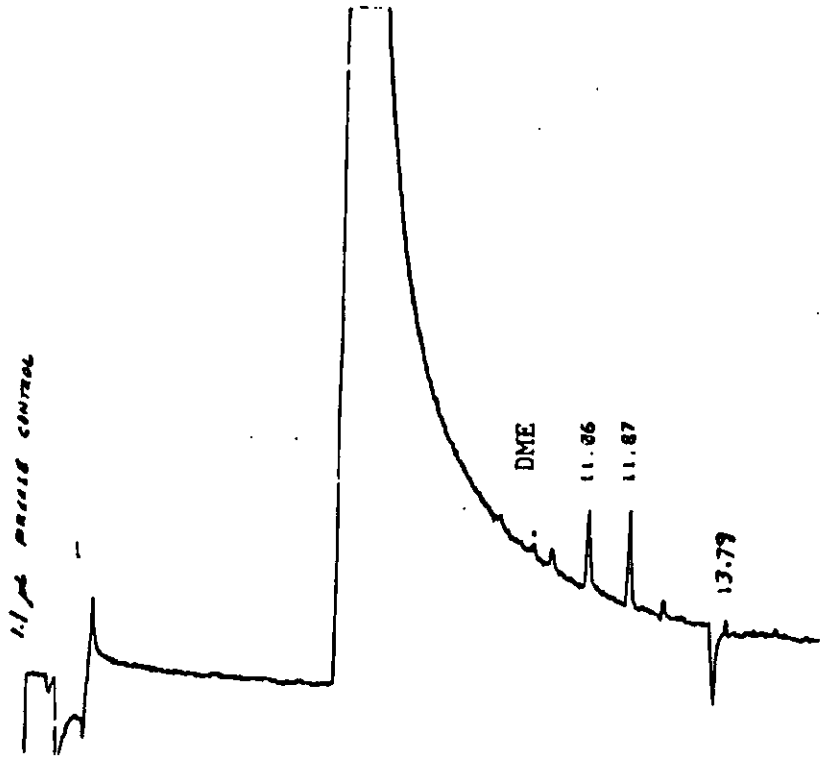
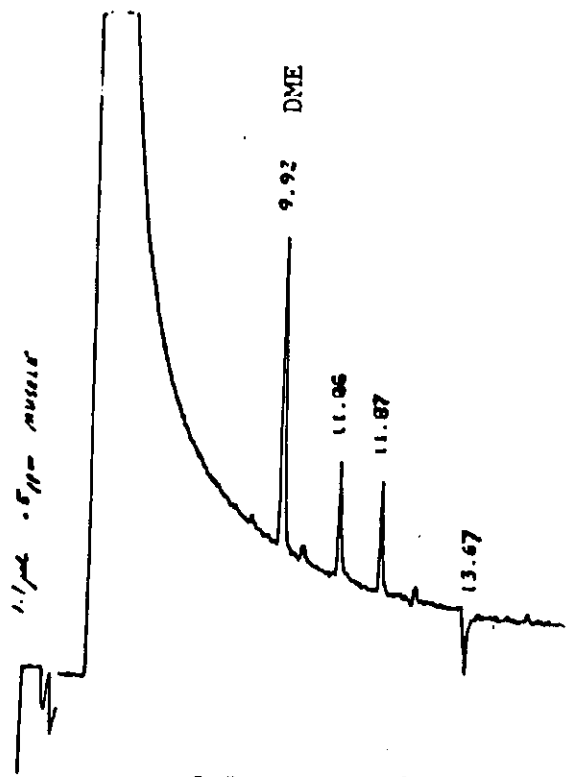


FIGURE 2

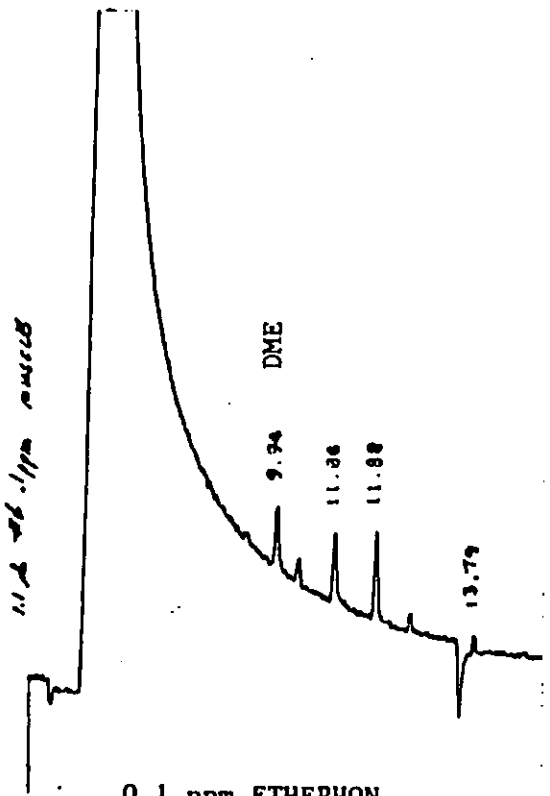
REPRESENTATIVE CHROMATOGRAMS FOR ANALYSIS OF MUSCLE TISSUE



CONTROL MUSCLE



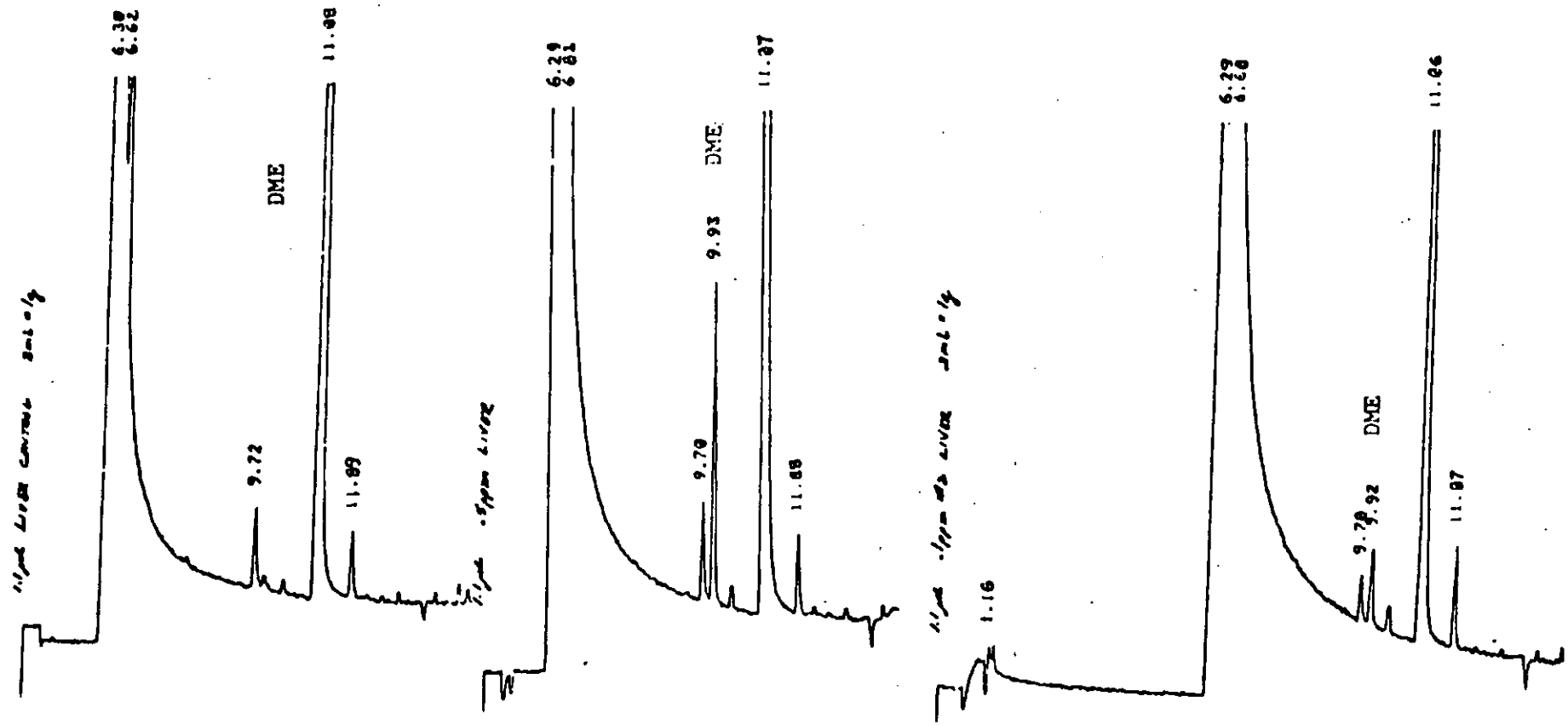
0.5 ppm ETHEPHON
FORTIFIED MUSCLE



0.1 ppm ETHEPHON
FORTIFIED MUSCLE

FIGURE 3

REPRESENTATIVE CHROMATOGRAMS FROM ANALYSIS OF LIVER TISSUE



CONTROL LIVER

0.5 ppm ETHEPHON
FORTIFIED LIVER

0.1 ppm ETHEPHON
FORTIFIED LIVER

FIGURE 4

CHROMATOGRAMS OF DIMETHYL ETHEPHON STANDARD AND CONTROL KIDNEY

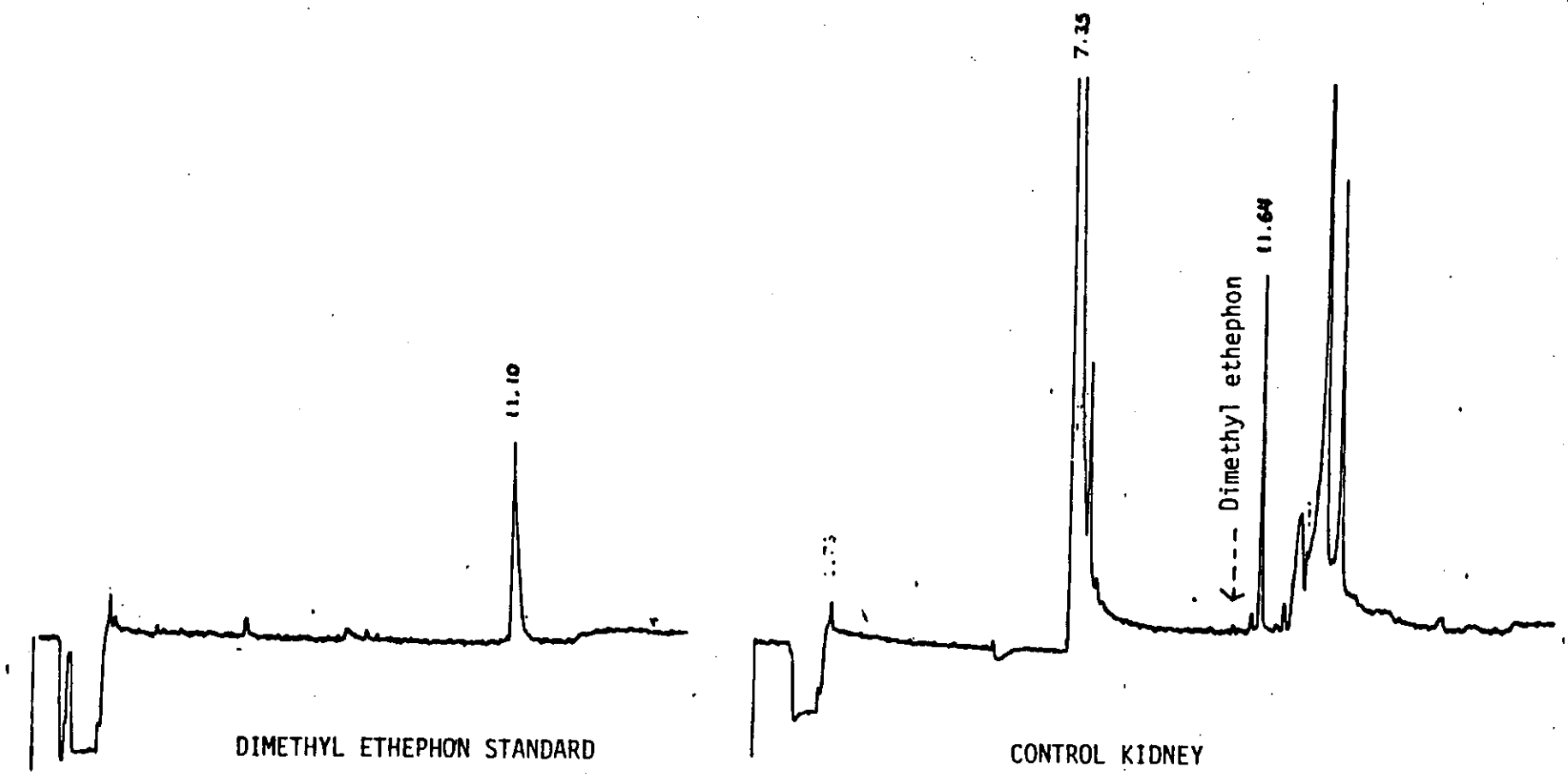


FIGURE 5

CHROMATOGRAMS OF EXTRACTS OF KIDNEY TISSUE FORTIFIED AT
0.1 AND 0.5 PPM LEVELS WITH ETHEPHON

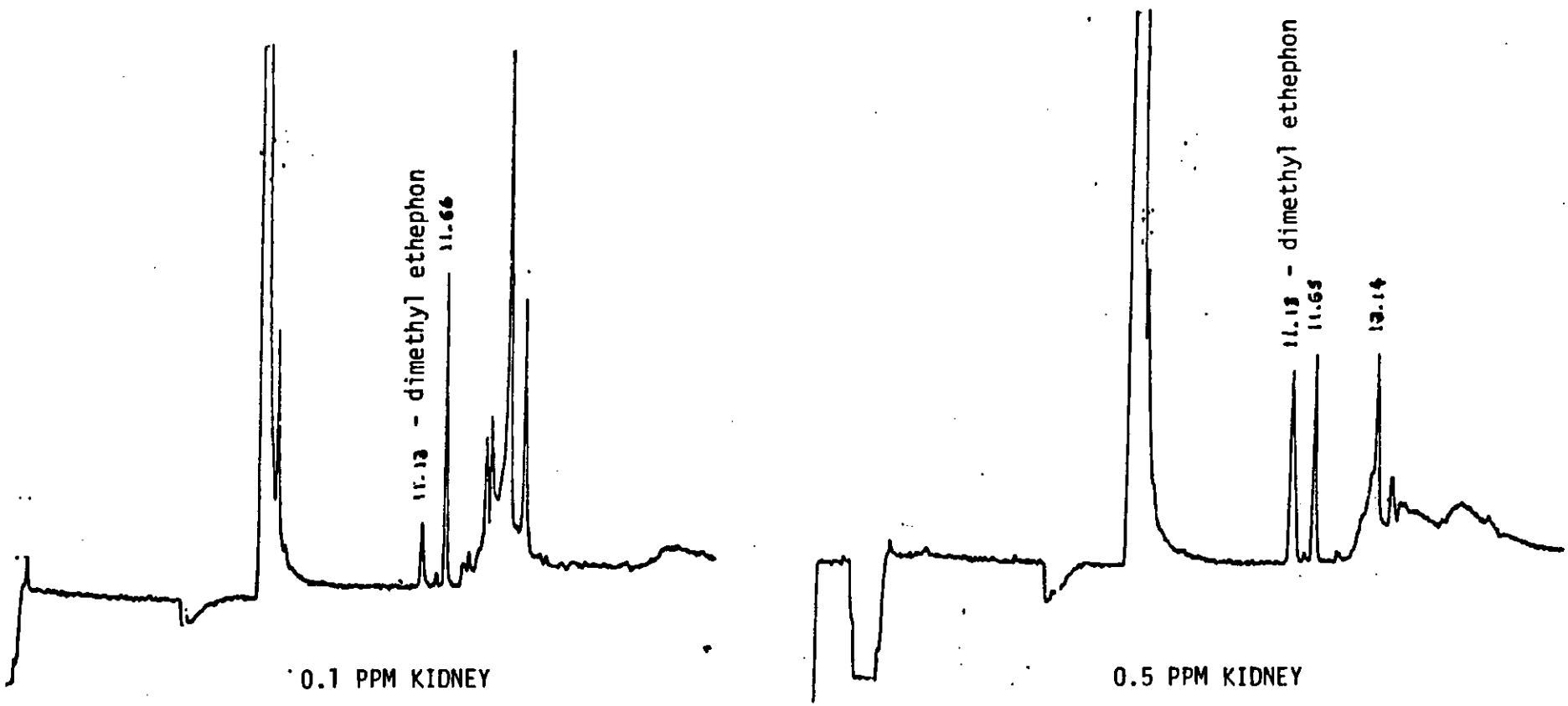


FIGURE 6

CHROMATOGRAMS OF DIMETHYL ETHEPHON STANDARD AND CONTROL MILK

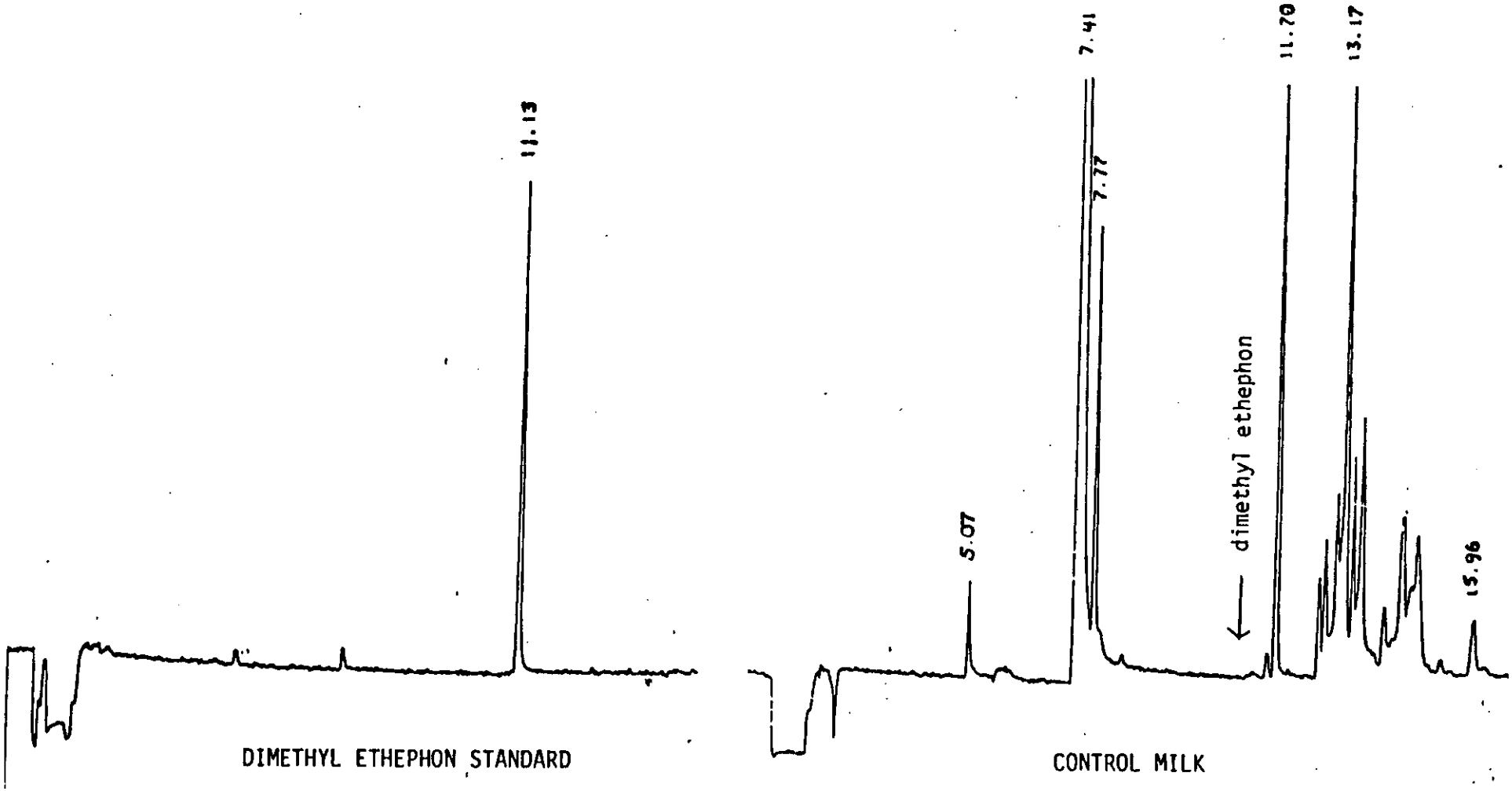
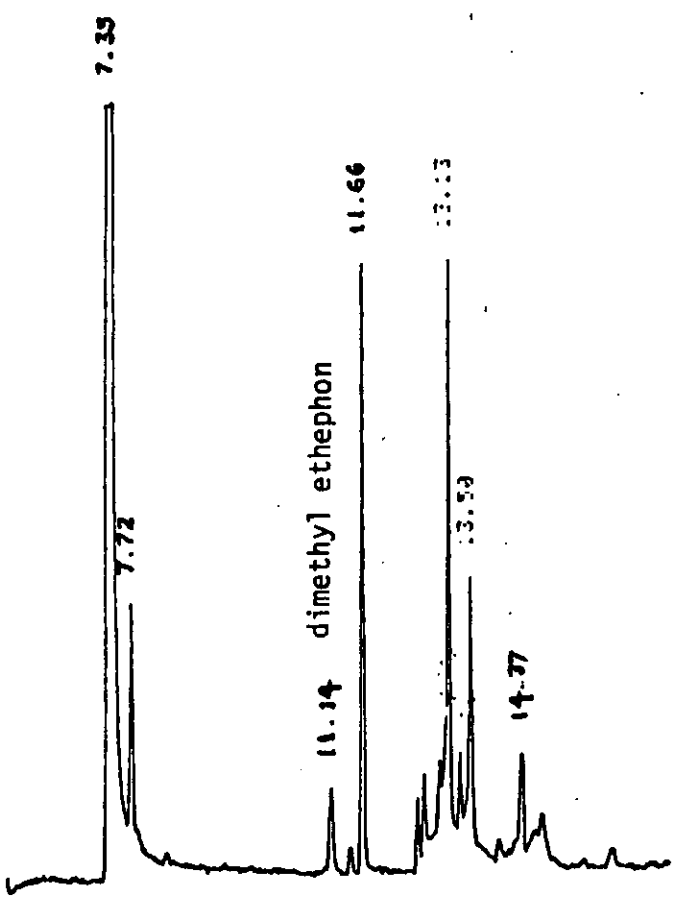
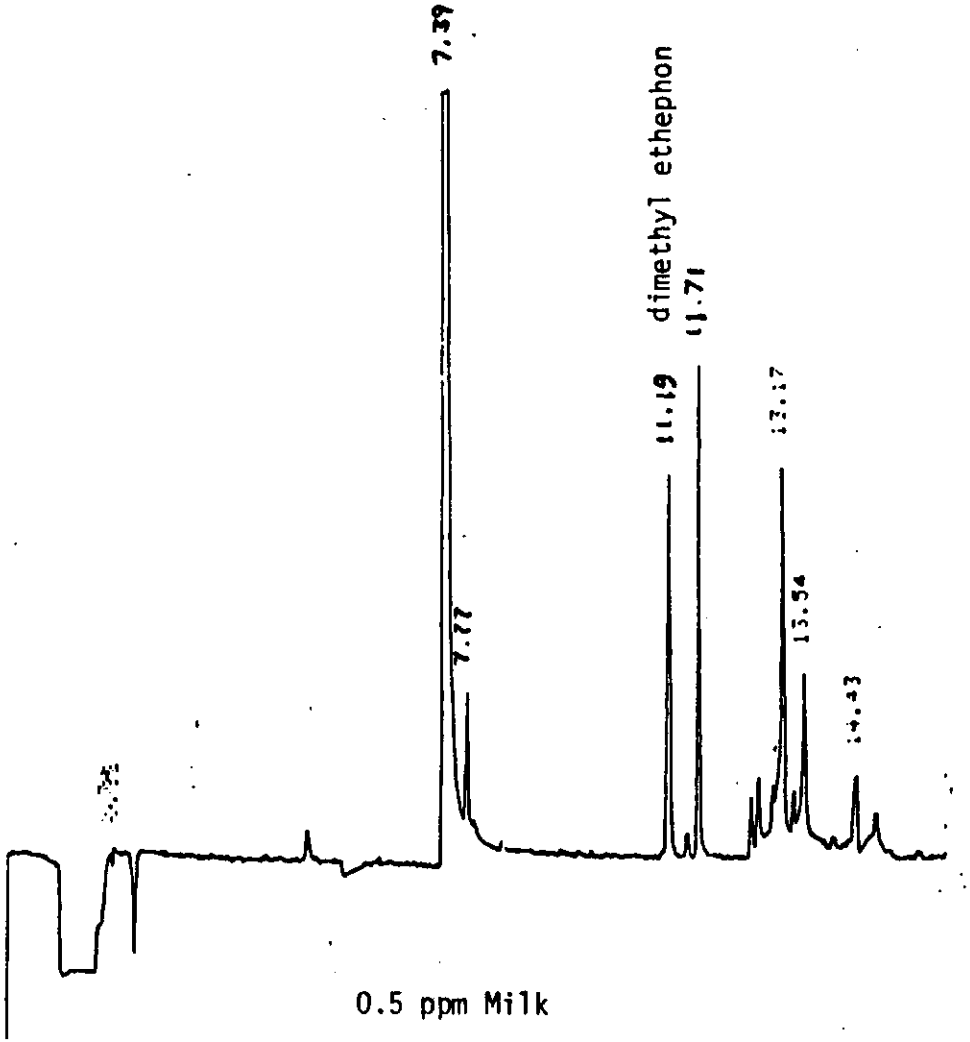


FIGURE 7

CHROMATOGRAMS OF EXTRACTS OF MILK FORTIFIED AT 0.1 AND 0.5 PPM LEVELS WITH ETHEPHON



0.1 ppm Milk



0.5 ppm Milk

CHROMATOGRAMS OF EXTRACTS OF CONTROL FAT AND FAT TISSUE
FORTIFIED AT 0.1 AND 0.5 PPM LEVELS WITH ETHEPHON
(CAPILLARY COLUMN)

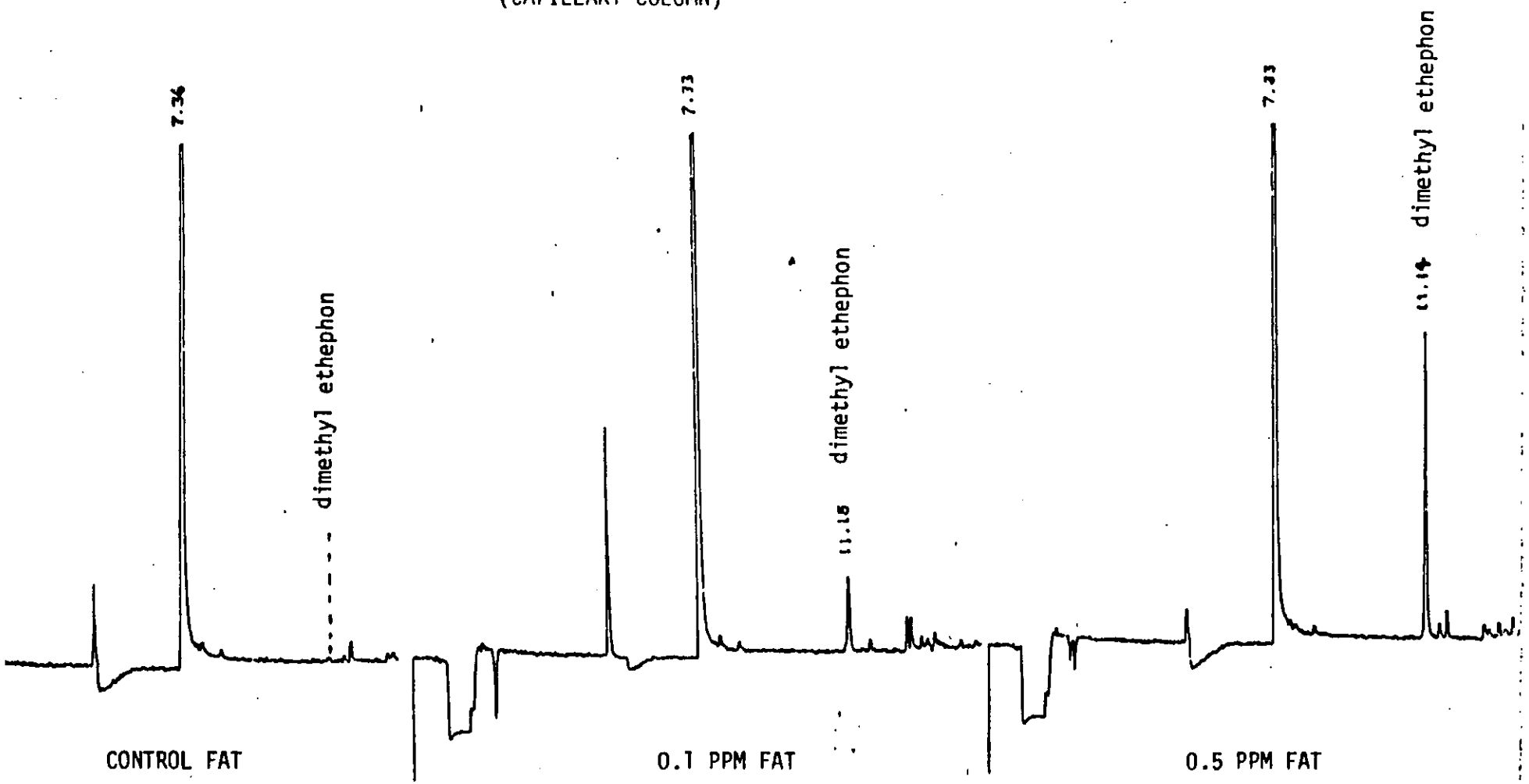


FIGURE 9

CHROMATOGRAMS OF EXTRACTS OF CONTROL FAT AND FAT TISSUE FORTIFIED AT 0.1 AND 0.5 PPM LEVELS WITH ETHEPHON (PACKED COLUMN)